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Application For Renewal of Research Grant
(Use extra pages as needed)

First Renewal ☐ Second Renewal ☐ Date: July 11,1975

1. Principal Investigator (give title and degrees):

Irene Y. Wang, Ph.D.
Assistant Professor

2. Institution & address:

College of Medicine
Medical University of South Carolina
80 Barre Street, Charleston, S. C. 29401

3. Department(s) where research will be done or collaboration provided:

Department of Basic and Clinical Immunology and Microbiology

4. Short title of study:

Genetic Differences in the In Vitro Metabolism of Chemical Carcinogens by Human and Mouse Tissues.

5. Proposed renewal date: Jan. 1, 1975

6. How results to date have changed earlier specific research aims:

Yields of individual metabolite of benzo(a)pyrene (BaP) catalyzed by microsomal enzymes of C57B1/6 (B6) and C3H mice are increased to different extents upon pretreatment with BaP, with 7,8-diol being generally increased the most in tissues tested which include liver, lung, spleen and kidney. For DBA/2 (D2) mice, pretreatment with BaP has no effect on liver, the induced enzyme activity in extrahepatic tissues of D2 mice are still lower than those of the corresponding tissues of untreated B6 or D2 mice. The preferential increase in the yield of 7,8-diol may be significant in the tumoregenesis in lungs of susceptible mice, since we reported (1) that 7,8-diol can be further activated by hamster liver microsomal enzymes to bind DNA. It is proposed to add the study of microsomal enzyme activity in various mouse tissues, especially lung, in further activating 7,8-diol to reactive forms.

7. How results to date have changed earlier working hypothesis:

The results to date tend to strengthen the original working hypothesis that there may be a correlation between the metabolite patterns of polycyclic aromatic hydrocarbons(PAH) and the susceptibility of animals to PAH-induced tumoregenesis. In the case of BaP metabolism by different strains of mice, the greater increase in the yield of 7,8-diol may be very important, and this aspect needs to be further studied as proposed above.

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8. Any additional facilities now required? Describe briefly:

Fraction collector: for collecting DNA fractions after CsCl gradient centrifugation of DNA incubated with metabolites of ^{3}H-BaP or of ^{3}H-3-MC.

Nitrogen evaporator: originally two nitrogen evaporators were available in handling large number of sample each time. Dr. Rasmussen transferred one of them to U.C. Irvine.

Cigarrette-smoking machine: for testing effect of cigarrette smoke inhalation on mouse tissue microsomal enzyme activities in produing various metabolites of BaP or 3-MC.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Due to transfers of professionals involved in the original proposal, Dr. R. E. Rasmussen and Dr. N. L. Petrakis are not participating the the continuation of this project.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

1. Wang, I. Y., Rasmussen, R. E. and Crocker, C. C., Metabolism of benzo(a)pyrene by microsomes from tissues of pregnant and fetal hamsters. Life Science 15, 1219 (1974).

2. Wang, I. Y., Metabolism of benzo(a)pyrene by tissue microsomes from different strains of mice. Proc. Am. Assoc. Cancer Res. 16, 377 (1975).

3. Rasmussen, R. E. and Wang, I. Y., Metabolism and binding of ^{3}H-benzo(a)pyrene to DNA in the presence of liver enzymes from rats treated with benzo(a)pyrene or phenobarbital.

4. Wang, I. Y., Rasmussen, R. E., Petrakis, N. L., Jan, C. C. and Wang, A. C. Enzyme induction and the difference in the metabolite pattern of benzo(a)pyrene produced by various strains of mice. Symposium on Polynuclear Aromatic Hydrocarbons. Battelle Columbus Lab. Oct. 1975.

12. Summary progress report (append in standard form as separate document, unless recently submitted).

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13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Irene Y. Wang, Ph.D.	40%	----
An-Chuan Wang, Ph.D.	5%	----
Technical		
Lab specialist I (to be recruited)	100%	11,760.00
Lab Specialist I (to be recruited)	100%	11,760.00

(All salaries include 8% increase for projected cost of living and merit raises).

Sub-Total for A 23,520.00

B. Consumable supplies (by major categories)	
Animals	3,000.00
Radioisotopes	2,500.00
Glassware	500.00
Chemicals (NADPH, etc.)	3,000.00
Scintillation counting vials	3,000.00
TLC plates	300.00

Sub-Total for B 12,300.00

C. Other expenses (itemize)	
Medical illustration and publication	1,000.00
Equipment maintance, contract and contracting	1,000.00
Travel	1,500.00
Misc. office supplies	300.00

Sub-Total for C 3,800.00

Running Total of A + B + C 39,620.00

D. Permonent equipment (itemize)	
Nitrogen Evaporator	500.00
Gilson microfraction collector	1,100.00

Sub-Total for D 1,600.00

E. Indirect costs (15% of A+B+C) 5,943.00

Total request 47,163.00

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Role of Metaboli Activation in Chemical Carcinogenesis	South Carolina State Approperiation for Research	$5000.00	July 1, 1975 to June 30,. 1976.

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Irene Y. Wang, Ph.D.

Signature Irene Wang Date July 11, 19

Telephone 803 792-4421
Area Code Number Extension

Checks payable to

Medical University of South Carolina

Mailing address for checks: Mr. L. W. Michaelis
Director of Business Operations
Medical University of South Carolina
80 Barre Street
Charleston, South Carolina 29401

Responsible officer of institution

Typed Name Mr. John E. Wise

Title Vice President and Treasurer

Signature _____ Date _____

Telephone 803 792-4291
Area Code Number Extension

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10. Experimental Protocol for the Ensuing Year:

(1) Genetic studies on the metabolite patterns of BaP catalyzed by lung homogneates from C3H, D2 and B6; their cross and backcrosses.

Studies on the genetic differences in the metabolite pattern of BaP by liver homogenates of the above strains of mice are in progress. The optimal incubation conditions for the production of diols of BaP is being set up, since the precusors of the diols -- epoxides of BaP, or 7,8-diol itself after further metabolic activation may be the ultimate carcinogens of BaP. Data to date indicate that for liver homogenate incubation system of C3H, D2 or C3HxD2 mice, short incubation interval (e.g. 3 min.) and low homogenate protein concentrations (0.25-0.5 mg/ml) are favorable for avoiding secondary metabolism of BaP metabolites. Different tissues have different kinetic curves for the metabolite yields of BaP. Best incubation conditions using lung homogenates of mice will be determined, then host control of metabolite patterns of BaP will be studied.

(2) Further metabolic activation of BaP metabolites by mouse tissue preparations --- DNA-binding in vitro.

We reported previously that 7,8-diol can be further activated by hamster liver microsomes to bind DNA in vitro (1). Recently Sims et al (2) also reported that metabolic activation of BaP proceeds by a diol-epoxide. Therefore, we propose to study the enzyme activities in mouse lung and liver tissue preparations (homogenates or microsomes) in further activating metabolites of BaP (especially 7,8-diol) to active intermediates as indicated by extent of binding to DNA.

Radioactive metabolites of BaP will be prepared from large-scale incubation of ^{3}H-BaP or ^{14}C-BaP using liver microsomes from rats pretreated with 3-MC for 9,10-diol and 7,8-diol, and from control hamsters for 4,5-diol (3, 4). Other metabolites of BaP can be obtained with either rat or hamster liver microsomes as enzyme source. Metabolites of BaP will be separated by TLC as described before (4). For DNA-binding studies, 200 μg prepurified DNA in citrate buffer solution will be incubated with isolated radioactive metabolite of BaP in the presence of NADPH and mouse tissue preparations, at 37°C for 30 to 60 min. Incubation mixtures will then be extracted extensively with mixture of chloroform and isoamyl alcohol, then the aqueous phase dialyzed against citrate buffer solution, and subjected to CsCl density gradient centrifugation. Fractions will be collected and aliquoat from each fraction will be counted for radioactivity as indication of extent of DNA-binding of metabolite of BaP after further enzymic activation.

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(3) Effect of inhalation of cigarette smoke by mice on the metabolite patterns of BaP in vitro:

Cigarette-smoke inhalation by mice will be performed in similar ways as reported by Kouri et al (5), whose results indicated that pulmonary AHH activity of B6, C3H and D2 strains of mice could be induced about six hours post inhalation of one to seven cigarette consecutively. Metabolite patterns of BaP by mouse liver or lung tissue preparations after cigarette-smoke inhalation will be compared for possible correlation the production of certain metabolite(s) of BaP and the susceptibility of lung tissues to BaP-induced tumors.

(4) Modification of metabolite patterns of BaP by fractions of cigarette-smoke condensate:

Some fractions of cigarette-smoke condensate have been reported to induce pulmonary AHH activity, and also inhibit BaP metabolism in vitro, e.g., B_1a, B_1b and N_{NM} (5). Metabolite patterns of BaP and DNA-binding by BaP in incubation system employing mouse tissue preparations will be studied in paralel with the presence of different fraction of condensate in the incubation mixture.

(5) Identification of metabolites of 3-MC catalyzed by liver and lung microsomes form C3H, B6 and D2 mice.

Incubation system for metabolism of 3-MC will be similar to that used for BaP, and extraction as well as separation of metabolites on TLC plates will be carried out as for BaP. UV spectrum of each isolated metabolite of 3-MC will be used for identification.

References:

(1) Borgen, A., Darvey, H., Castagnoli, N., Crocker, T., Rasmussen, R. and Wang, I., J. Med. Chem., 16, 502(1973).
(2) Sims, P., Grover, P. L., Swaisland, A., Pal, K. and Hewer, A., Nature, 252, 326(1974).
(3) Rasmussen, R. E. and Wang, I. Y., Cnacer Res., 34, 2290(1974).
(4) Wang, I. Y., Rasmussen, R. E. and Crocker, T. T., Biochem. Biophys. Res. Commun. 49, 1142(1972).
(5) Kouri, R. E., Demoise, C. E. and Whitmire, C. E., Exp. Respiratory Carcinogenesis and Bioassays, Springer-Verlag, 1974.

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Source: https://www.industrydocuments.ucsf.edu/docs/tlpl0000

Progress Report: Jan. 1, 1975 to June 30, 1975.
CTR Grant #1005
Irene Y. Wang, Ph.D.
Dept. Basic and Clinical Immunology and Microbiology
Medical University of South Carolina
Charleston, South Carolina 29401

Genetic Differences in the In Vitro Metabolism of Chemical Carcinogens by Human and Mouse Tissues.

A. Results:

(I) Effect of BaP-pretreatment on the in vitro metabolite pattern of BaP by mouse tissue microsomes.

To date, five strains of mice have been tested: C57Bl/6 (B6), C3H, DBA/2 (D2), B6xD2 and C3HxD2. Mice were pretreated with BaP (2 mg/100 g body weight) 24 hrs before sacrifice, control mice were injected with corn oil only. Microsomes were prepared from liver, lung, kidney and spleen, protein concentrations were determined by Lowry's method(2). Metabolism of BaP was studied with freshly prepared microsomes. Incubation system consisted of 1 μg/ml ^{3}H-BaP (specific activity 25 ci/mmole), 1 mg/ml NADPH, 0.25 mg/ml microsomal protein in sodium citrate buffer (1). Incubation was carried out at 37°C from three to ten minutes under subdued light. Incubation mixture was extracted twice with 2 volume ethyl acetate, and the organic layer was dried under nitrogen. The residue was redissolved in acetone-ethyl acetate mixture and spotted on TLC plate. Separation of metabolites of BaP was done by chromatography with benzene alone for the detection of BaP-4,5-epoxide, or first with benzene then immediately in the same direction with benzene:ethanol(95:5). Identification of metabolites of BaP was by comparison with authentic compounds co-chromatographed with samples, radioscannings as well as by UV absorption spectra of metabolites separated from large-scale incubation mixtures. Quantitation of metabolites of BaP was made by scintillation counting of strips containing radioactive spots.

(a) Qualitative comparison of metabolite patterns of BaP.

Figs.1, 2, and 3a show the separation of metabolites of BaP catalyzed by liver microsomes from D2, C3H and B6 mice respectively. The metabolites are: peak (a), origin of TLC and more-polar metabolites of BaP; peak (b), 9,10-diol; peak (c), 7,8-diol; peak (d), 4,5-diol; peak (e), 3-OH and other phenols of BaP; peak (f), not identified; peak (g), quinones of BaP; peak (h), BaP-4,5-epoxide; peak (i), unmetabolized BaP.

With liver microsomes of D2 mice, relative peak heights for control and pretreated mice (Fig. 1) are almost identical. With C3H (Fig. 2) and B6 (Fig. 3a) mouse liver microsomes, every

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metabolite was increased due to pretreatment with BaP. The largest increase was at the peak for 7,8-diol. Fig. 3b. shows the separation of BaP-4,5-epoxide from BaP and other metabolites of BaP on TLC plate by chromatography with benzene only.

(b) Quantitative comparison of metabolite yields of BaP.

In Figs. 4, 5, 6, 7, the hedged columns represent results from control mice, and the hollow bars the results from BaP-pretreated mice). As can be seen in Fig. 4, which includes the results from B6xD2 and C3HxD2 mouse liver microsomal incubation systems also, livers of B6 and B6xD2 mice showed great response to hydrocarbon-induced enzyme activities in metabolizing BaP, while increase in the yields of BaP metabolites in pretreated C3H mouse liver was smaller, and D2 mouse liver did not respond to pretreatment with BaP in the enzyme activities in metabolizing BaP. Results to date with C3HxD2 mouse liver microsomes are not clear cut, more studies on individual mouse of this hybrid atrain will be carried out. However, it is very obvious that the greatest increase due to pretreatment with BaP was the yield of 7,8-diol, in the cases with B6, B6xD2 and C3H mouse livers.

For extrahepatic tissue microsomal incubation system for metabolizing BaP, protein concentration was 1.0 mg/ml and incubation for 10 min. All the other conditions were the same as for liver microsomal incubation system. As can be seen in Table 1, 2, or 3, (or Fig. 5, 6 or 7, for comparison of yields of metabolites of BaP by extrahepatic microsomes), metabolite patterns of BaP by C3H and B6 lung microsomes were very similar, as well as the effect on BaP-pretreatment on the enzyme activities in metabolizing BaP by these lung microsomes. D2 strain of mice showed very little response to BaP-pretreatment, there was a small increase in metabolite yields of BaP by lung microsomes of pretreated D2 mice, but the yields were still much lower than those produced by lung microsomes of control B6 or C3H mice. The significance of the preferential increase in the yield of 7,8-diol by lung microosmes of B6 or C3H mice warrent further detailed studies.

(2) Incubation system with homogenates of mouse livers:

Frozen mouse liver pieces were supplied by Dr. R. E. Kouri. Each liver piece was weighed and homogenized in 20 times of TKM-sucrose buffer (1). Protein concentrations were determined by Lowry's method(2). Each incubation sample contained one to four mg/ml homogenate protein in 1 ml TKM-sucrose buffer, 1 μg/ml ^{3}H-BaP (specific activity 25 ci/mmole) in 50 μl acetone, 1 mg/ml NADPH, sodium citrate buffer (1) was added to make the final incubation volume to 2 ml. Incubation was at 37°C for 3 min. Extraction, separation, identification and quantitation of metabolites of ^{3}H-BaP were carried out in the same manner as for microsomal incubation system.

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Fig. 8 shows that metabolite patterns of BaP catalyzed by liver homogenates from control C3H, D2 and C3HxD2 mice are similar. At higher protein concentration (4 mg/ml), yields of quinones of BaP increased at the expense of phenols of BaP. Therefore, lower protein concentrations are preferred in avoiding secondary metabolism of metabolites of BaP. For Fig. 9, 1 mg/ml homogenate protein was used in incubation system. For mice pretreated with 150 µg 3-MC, livers of D2 mice did not show response to pretreatment; while C3H and C3HxD2 mouse livers showed similar response to 3-MC-pretreatment. Yield of 7,8-diol was increased to about 25-fold while that of 3-OH-BaP to about 4-fold.

Studies are in progress in determining the linear portion of the protein concentrations in the production of the diols for genetic studies using crosses and backcrosses of these strains of mice.

(3) Separation of Metabolites of 3-methylcholanthrene (3-MC):

Test runs were done by using microsomes from 60-day old Sprague Dawley rats, since we reported that BaP-pretreatment and phenobarbital (PB)-pretreatment caused different increase in the various metabolites of BaP (3). Incubations were similar to those used for BaP, 10 nmole/ml 3-MC was used in the incubation mixture and incubation was carried out for 10 min. Fig. 10 shows the radioscans of metabolites of 3-MC separated on TLC plate using the same solvent systems as for the separation of BaP metabolites. The metabolites of 3-MC need to be identified, however, this TLC system seems feasible for the separation of 3-MC metabolites catalyzed by mouse tissue microsomes or homogenates. Liver microsomes from PB-pretreated rats seemed to give higher yield of metabolite at position marked x in Fig. 10. Metabolite patterns of 3-MC by mouse liver microsomes will be studied in similar manner in the remaining portion of the current year.

(4) Metabolism of ^{3}H-BaP by Human Lymphocytes in Culture:

This protion of study was proposed in original grant proposal and was instructed to give priority to studies on mouse tissues upon approval of funding of this grant. The data reported here were results obtained previously. Lymphocytes prepared from human peripheral blood were cultured at 2×10^{6} cells/ml in RPMI 1640 medium supplemented with fetal calf serum and anti biotics. For mitogen stimulated lymphocytes, phytohemaagglutinin (PHA) or pokeweed mitogen(PWM) was added in strength of 0.1 ml/10 ml medium at the start of culture. ^{3}H-BaP 10 µci/ml, 25 ci/mmole) was added 24 hrs later. Culture was continued for another 24 or 48 hrs. Medium was then extracted with 2 volumes ethyl acetate and metabolites of ^{3}H-BaP were analyzed in the same manner as described previously. Metabolite patterns of ^{3}H-BaP by lymphocytes in culture 48 hrs after addition of ^{3}H-BaP are shown in Fig. 11, where peak (1),

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origin of TLC and more-polar metabolites of BaP; peak (2), not identified; peak(3), 9,10-diol; peak(4), 7,8-diol; peak(5), 4,5-diol; peak(6), 3-OH and other phenols of BaP; peak (7), quinones of BaP; peak(8), unmetabolized BaP. Panel A of Fig. 11 shows metabolite pattern of ^{3}H-BaP without any mitogens added to the culture medium; panel B shows that the presence of PHA in culture medium caused increase in the yield of phenols of BaP and decreased yields of diols. The quantitative data for the metabolite yields of BaP by lymphocytes in culture are shown in Fig. 12. No further studies on lymphocytes have been undertaken.

B. Plans for the remaining portion of the current year:

(1) Finish analysis data for optimal conditions for metabolism of BaP by liver homogenates, and then study the metabolite patterns of BaP with crosses and backcrosses of C3H, D2 and B6 strains of mice for genetic control of response to enzyme induction for the production of certain metabolites of BaP, especially the yields of the diols.

(2) Study the effect of pretreatment with various inducers of AHH activity on the metabolite patterns of BaP, e. g., the pretreatment of mice with phenobarbital, benzoflavones, fractions of cigarette smoke condensate.

(3) Column chromatography for different forms of cytochrome p-450, employing the method of Comai and Gaylor(4).

References:

1. Wang, I. Y., Rasmussen, R. E. and Crocker, T. T., Biochem. Biophys. Res. Commun., 49, 1142(1972).

2. Lowry, O. H., Rosebrough, N. A., Farr, A. L. and Randall, R. J., J. Biol. Chem. 193, 265(1951).

3. Rasmussen, R. E. and Wang, I. Y., Cancer Research, 34, 2290(1974).

4. Comai, K. and Gaylor, J., J. Biol. Chem., 248, 4947(1973).

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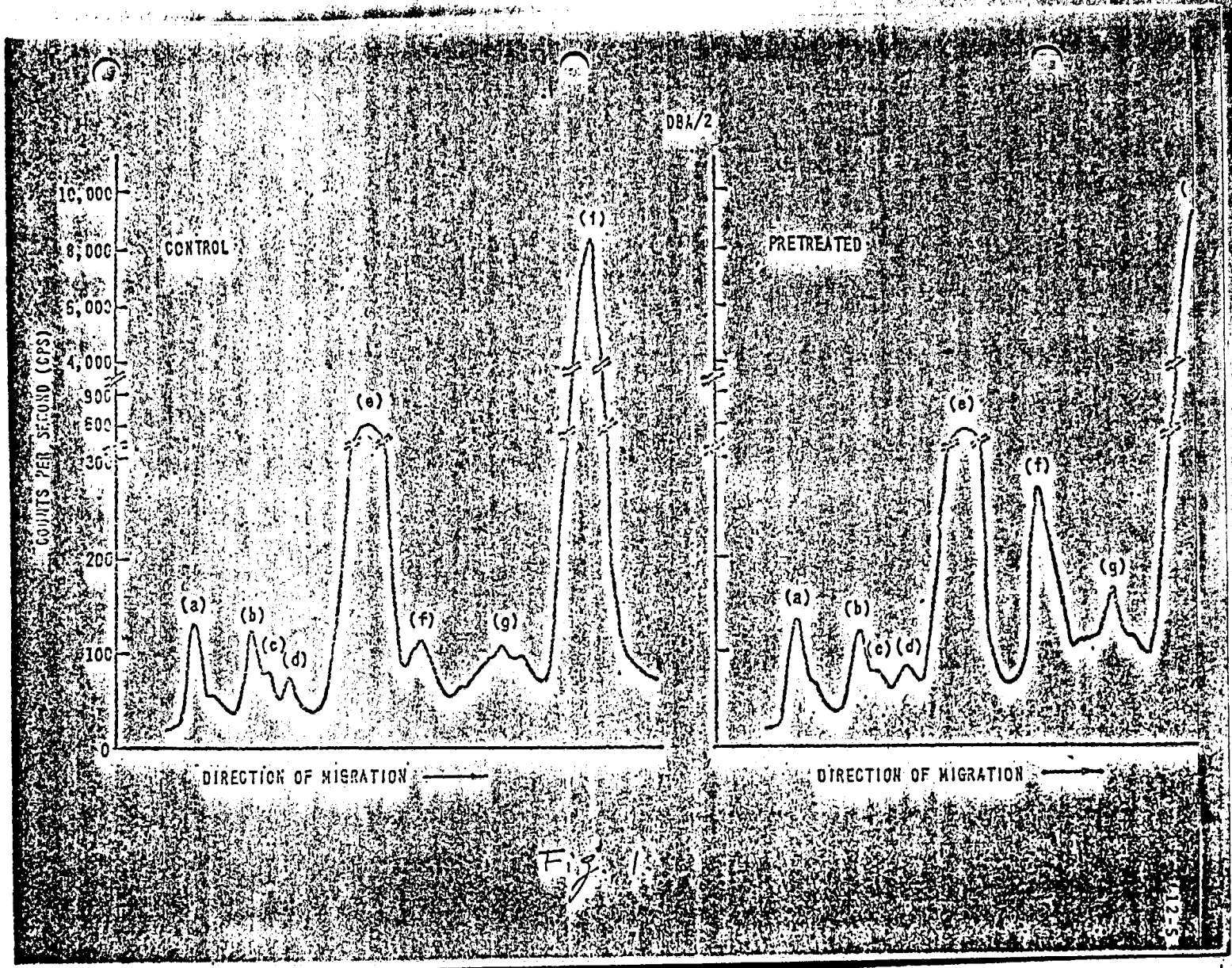

Fig. 1

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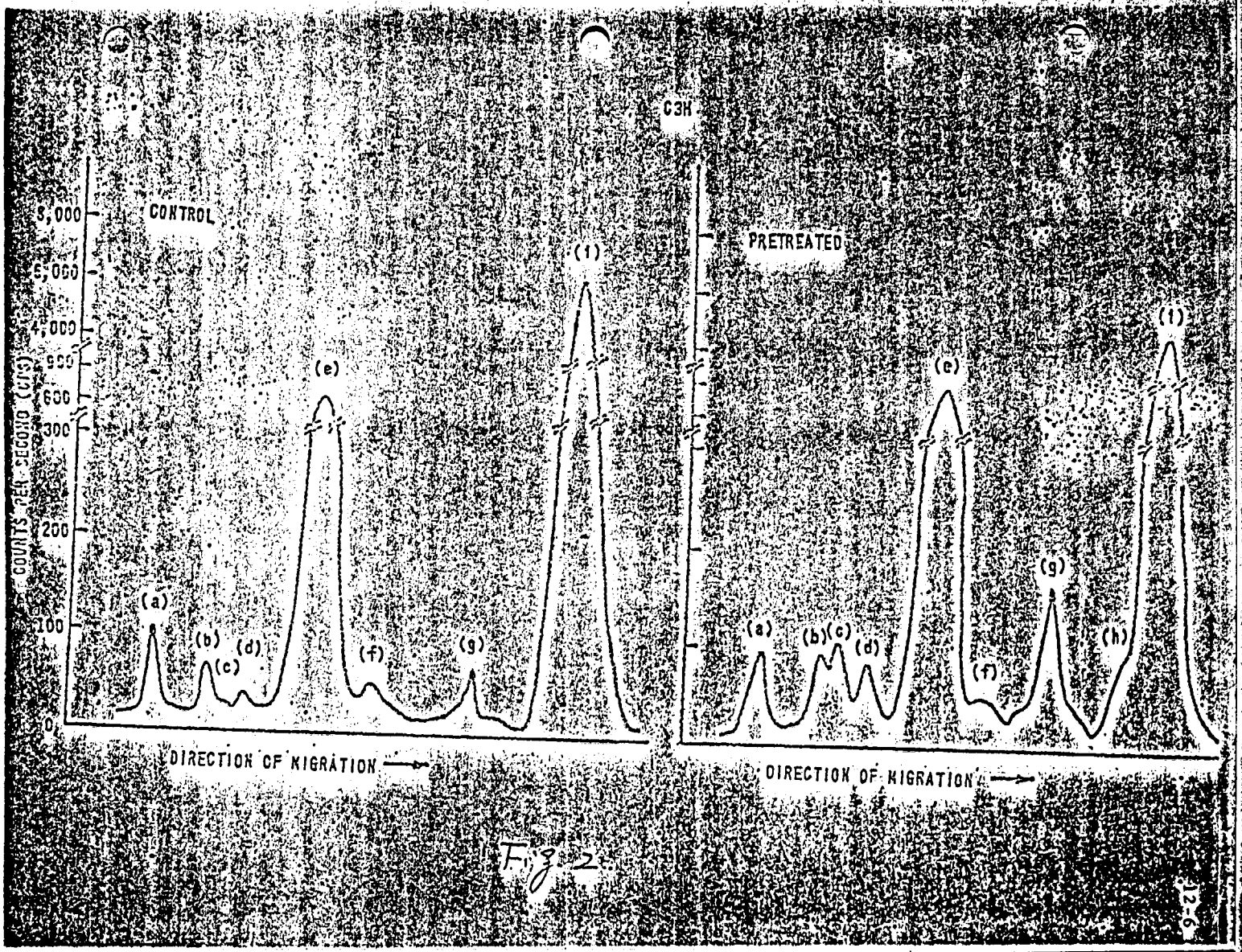

Fig. 2

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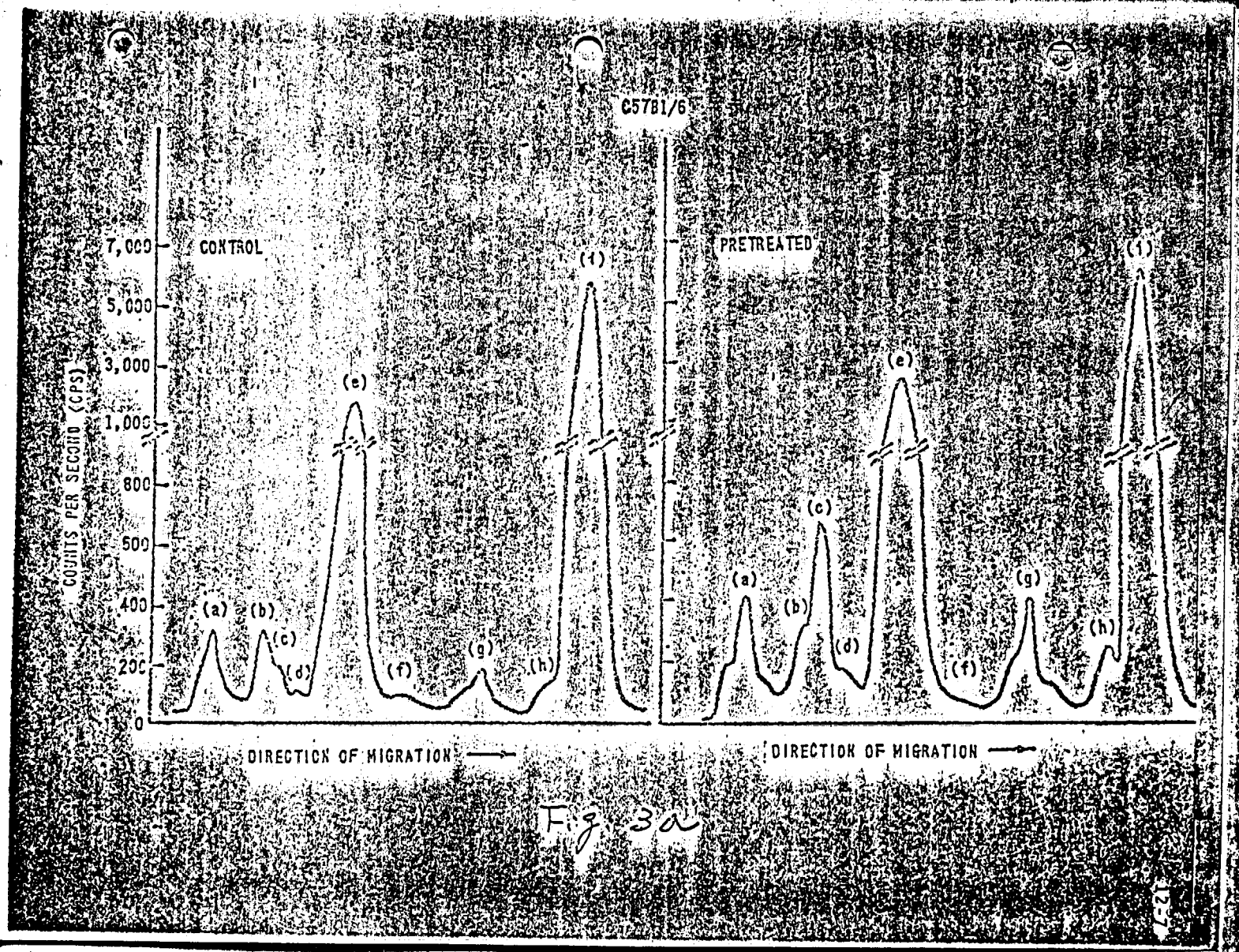

Fig. 3a

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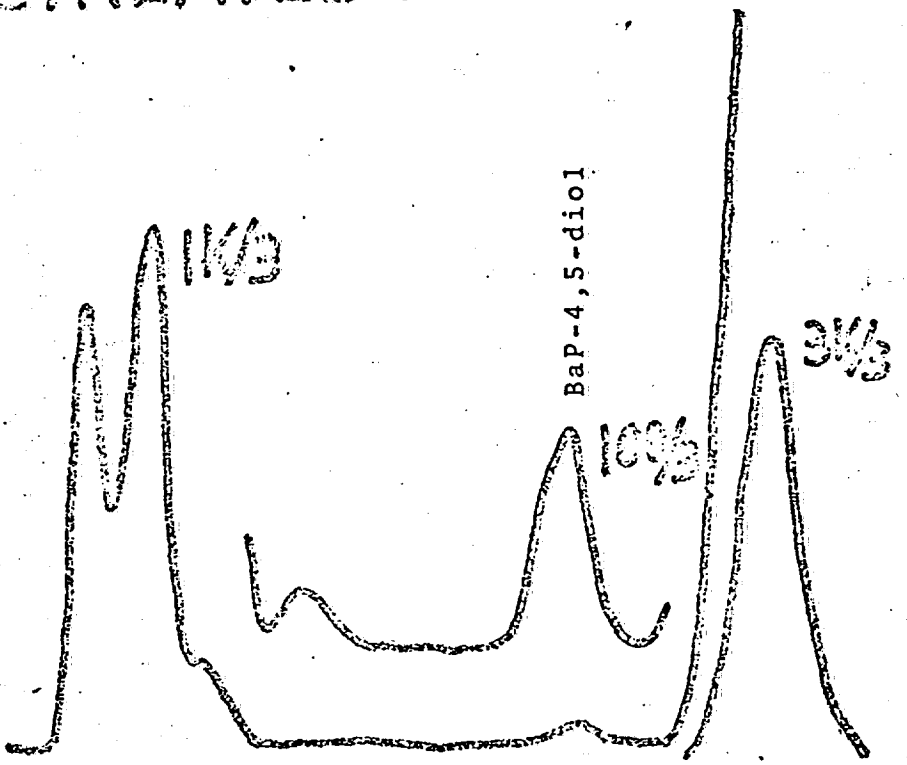
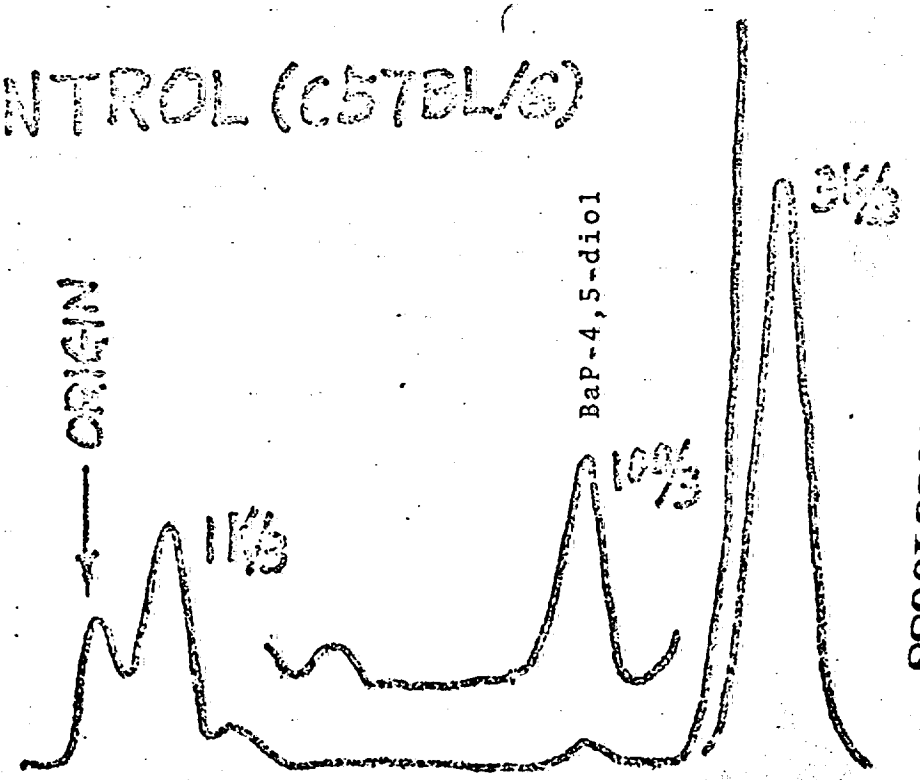
PRETREATED (C57BL/6)



CONTROL (C57BL/6)



DIRECTION OF MIGRATION →

Fig 3b.

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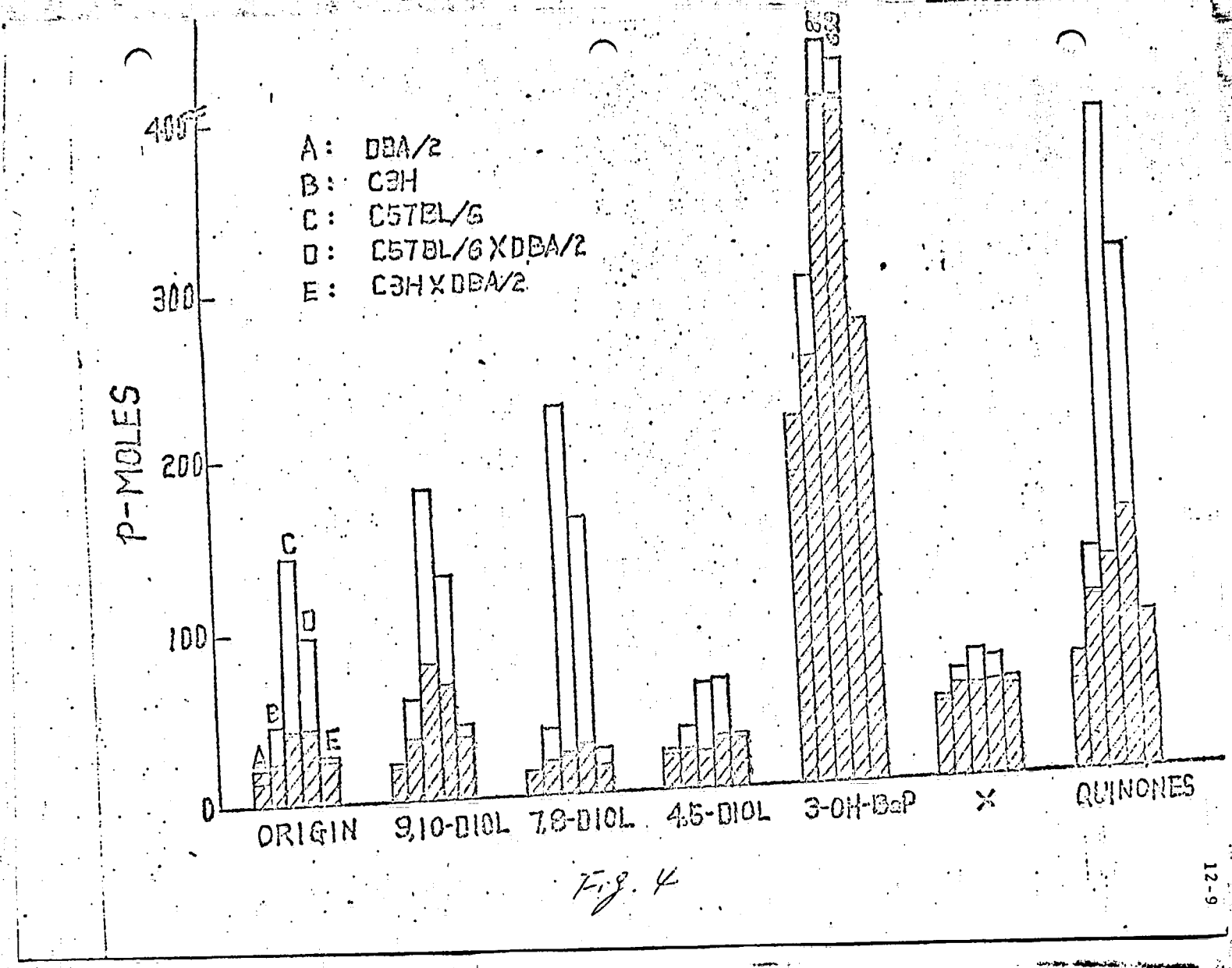

Fig. 4

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TABLE 1.

DBA/2 MICE

Metabolite	Lung		Kidney		Spleen		
	Control	Treated	Control	Treated	Control	Treated	Blank
Polar metabolites at origin of TLC	1.08	3.34	.59	.89	.95	1.58	2.03
9,10-diol	2.14	3.44	2.05	1.19	1.29	2.01	2.69
7,8-diol	2.97	8.82	2.20	1.34	2.18	4.43	1.12
4,5-diol	1.51	7.70	1.02	.84	1.02	.79	1.53
3-OH and other phenols of BaP	9.43	23.74	4.50	6.63	12.33	12.19	4.80
Quinones of BaP	90.28	91.80	99.37	96.74	102.27	98.91	73.98
Totals	107.42	138.84	109.74	107.64	120.06	119.90	86.16

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TABLE 2

C3H MICE

Metabolite	Lung		Kidney		Spleen		
	Control	Treated	Control	Treated	Control	Treated	Blank
Polar metabolites at origin of TLC	2.66	46.40	.61	1.66	.61	1.21	.69
9,10-diol	16.02	126.47	.37	10.77	.70	7.35	.24
7,8-diol	21.40	283.94	.73	12.73	2.92	12.72	.28
4,5-diol	4.39	62.35	.24	2.74	.76	1.35	.37
3-OH and other phenols of BaP	87.01	987.10	5.28	64.88	11.72	35.50	1.51
Quinones of BaP	38.00	349.29	8.33	22.33	15.99	19.66	9.10
Totals	169.48	1855.55	15.56	115.11	32.70	77.79	12.19

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Source: https://www.industrydocuments.ucsf.edu/docs/tlpl0000

TABLE 3

C57B1/6 MICE

Metabolite	Lung		Kidney		Spleen		
	Control	Treated	Control	Treated	Control	Treated	Blank
Polar metabolites at origin of TLC	5.07	42.04	7.44	11.98	2.07	3.75	1.51
9,10-diol	32.53	248.85	4.45	22.67	3.15	7.56	.47
7,8-diol	25.70	220.11	3.13	53.67	3.67	11.05	.58
4,5-diol	5.51	28.65	3.89	21.59	1.19	2.43	.93
3-OH and other phenols of BaP	121.62	1146.49	17.97	153.66	26.45	63.19	3.27
Quinones of BaP	52.87	338.52	153.12	191.86	40.12	42.38	48.75
Totals	243.30	2024.66	190.00	455.43	76.65	130.36	55.53

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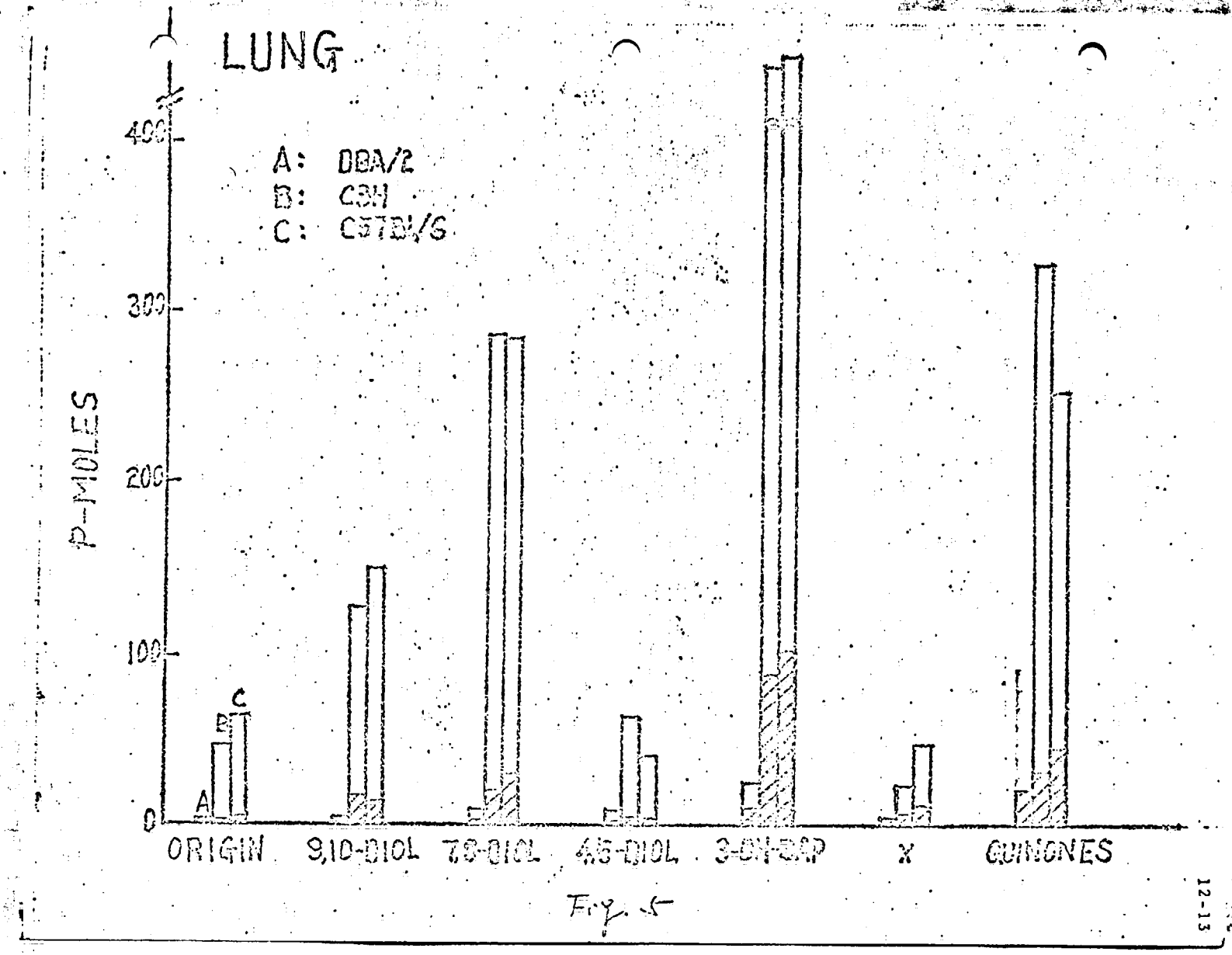

Fig. 4

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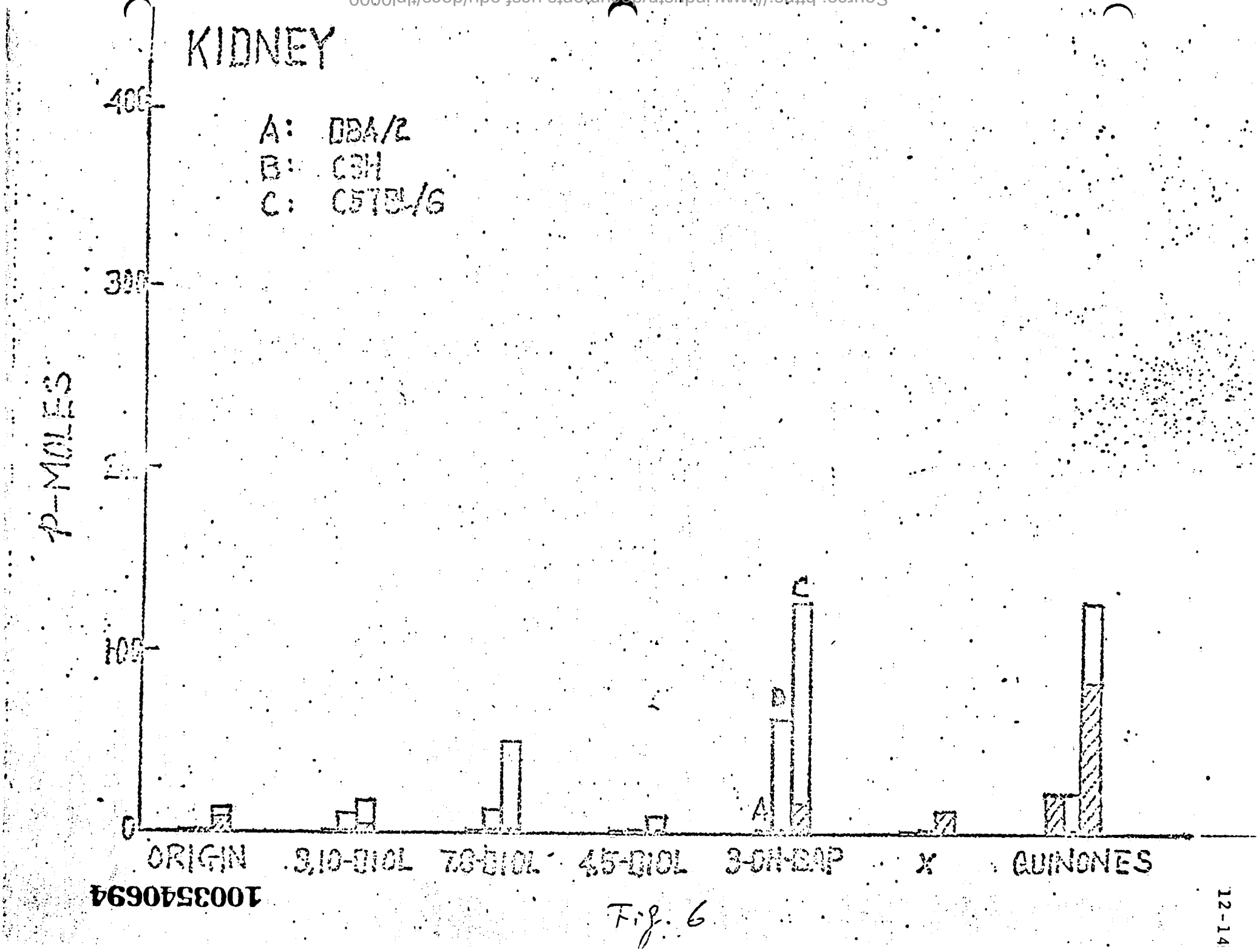

Fig. 6

1003540694

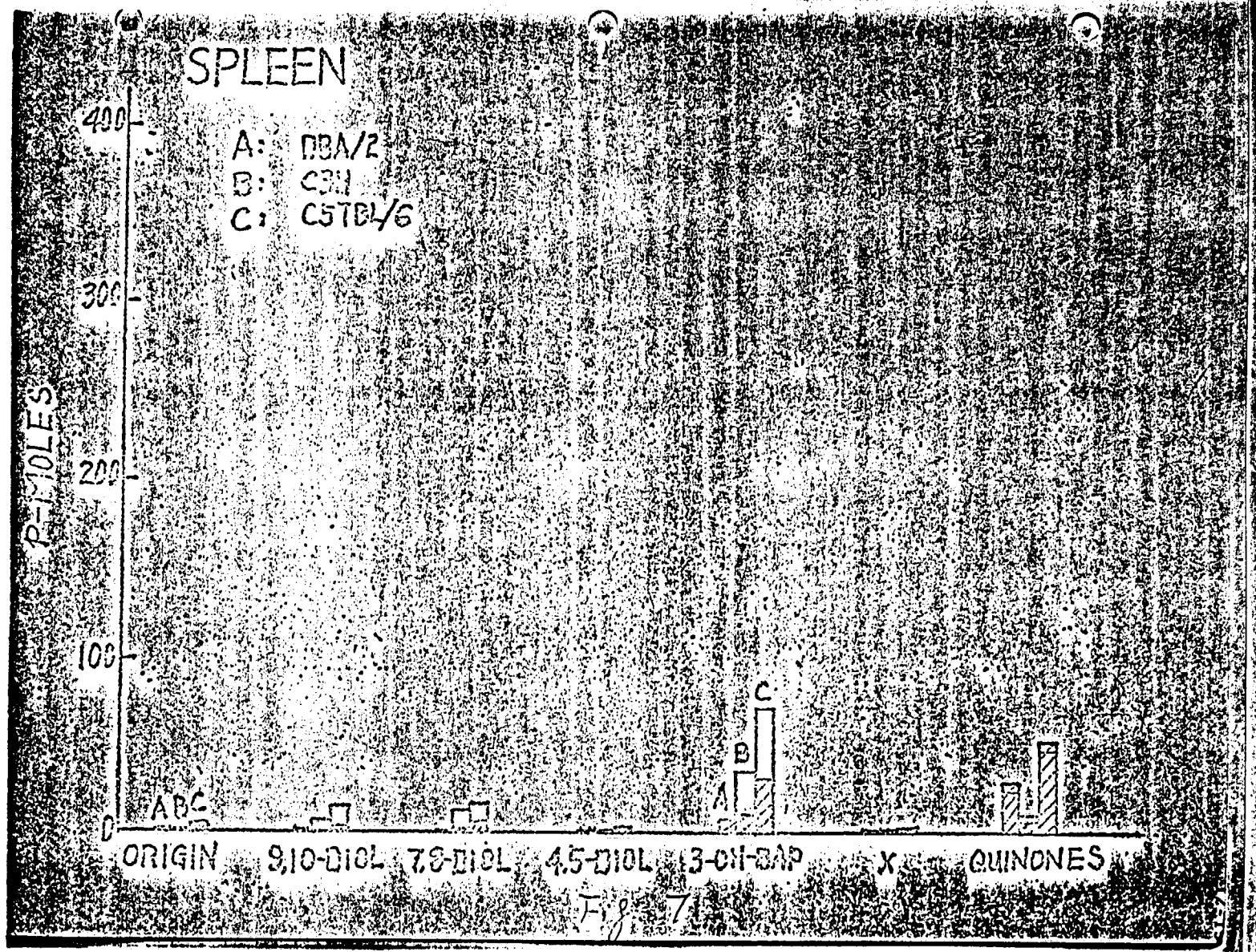

Fig. 7

1003540695

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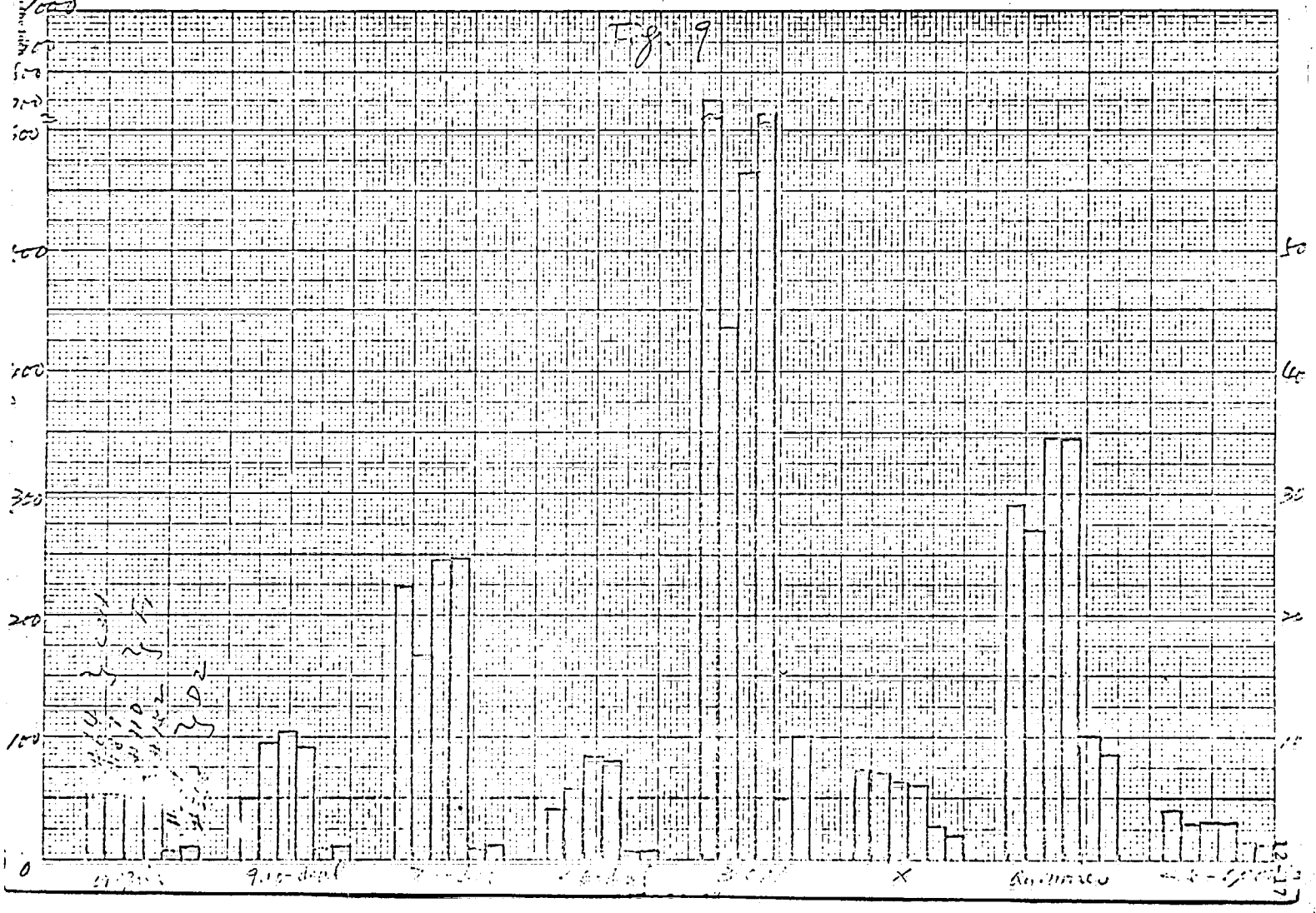

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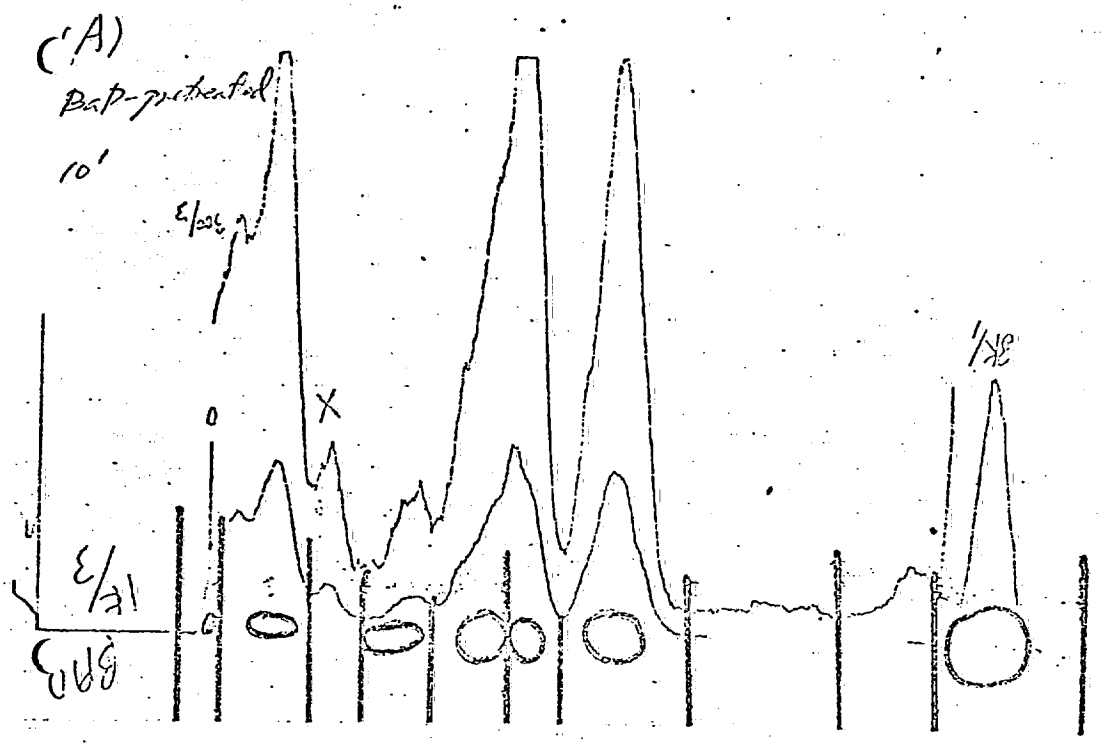

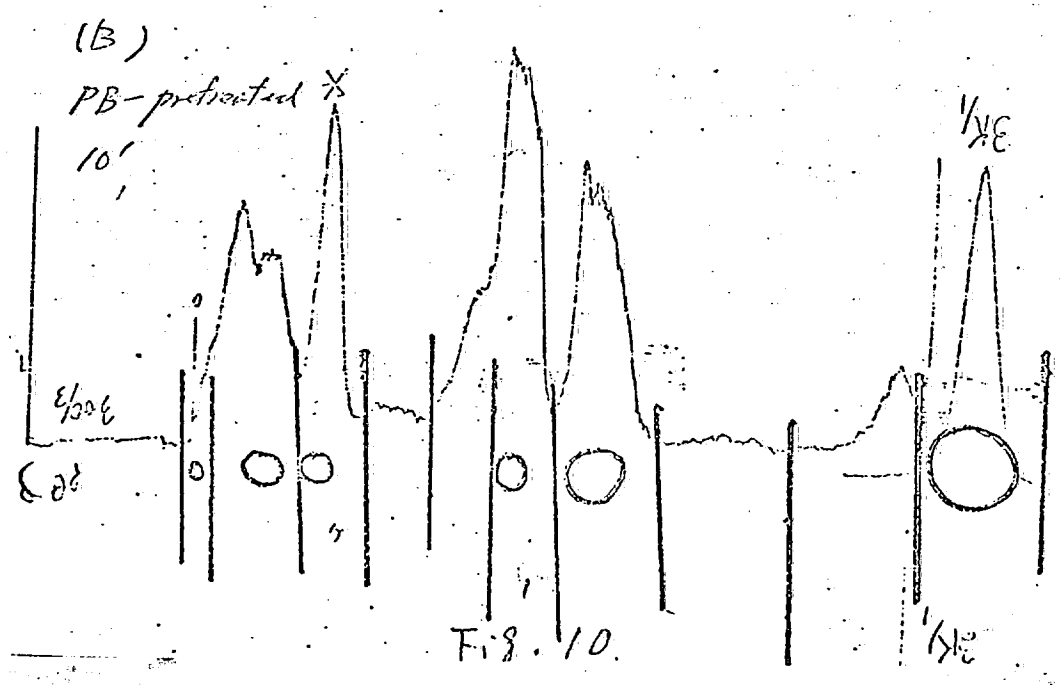

Fig. 10.

1003540698

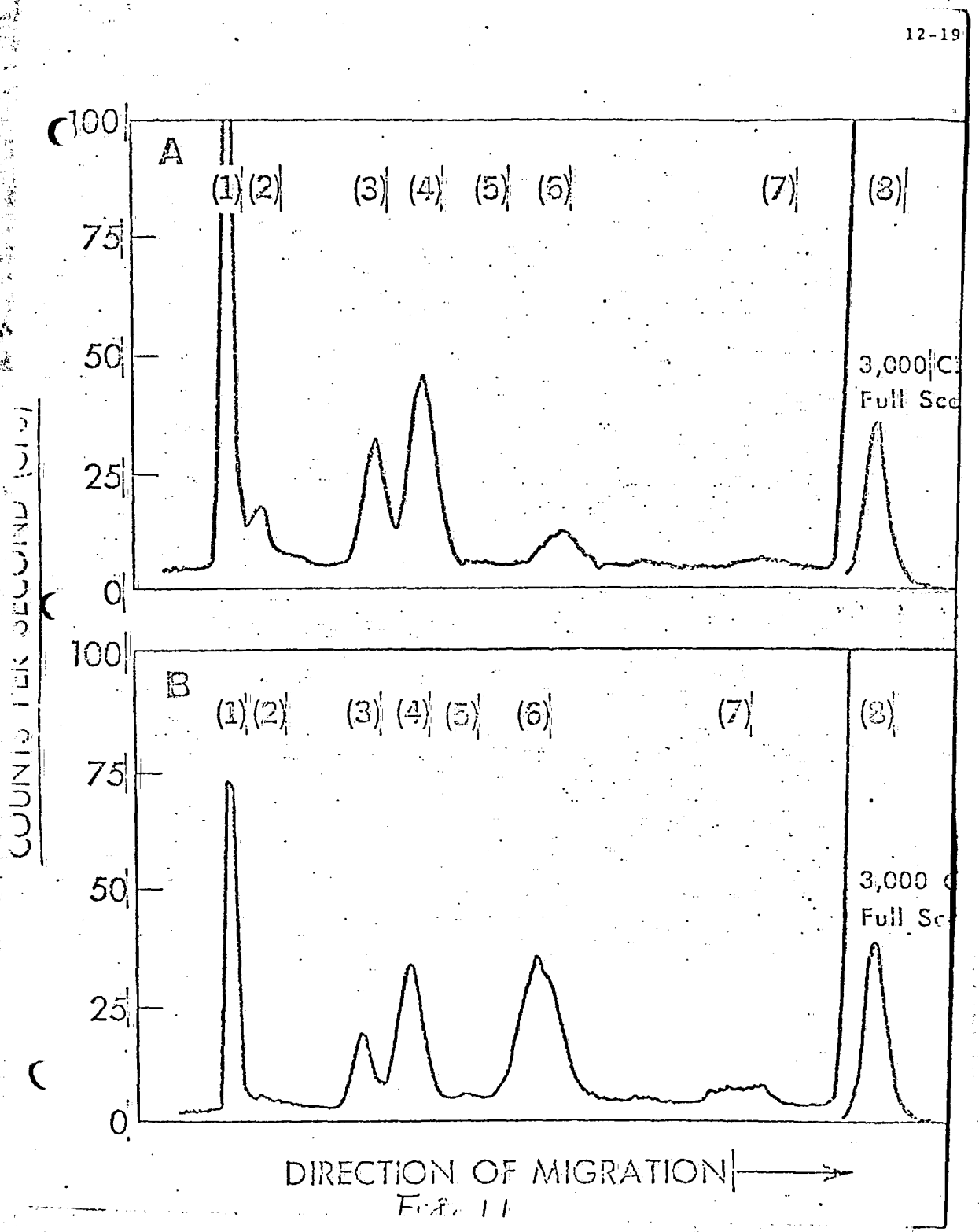

Fig. 11

1003540699

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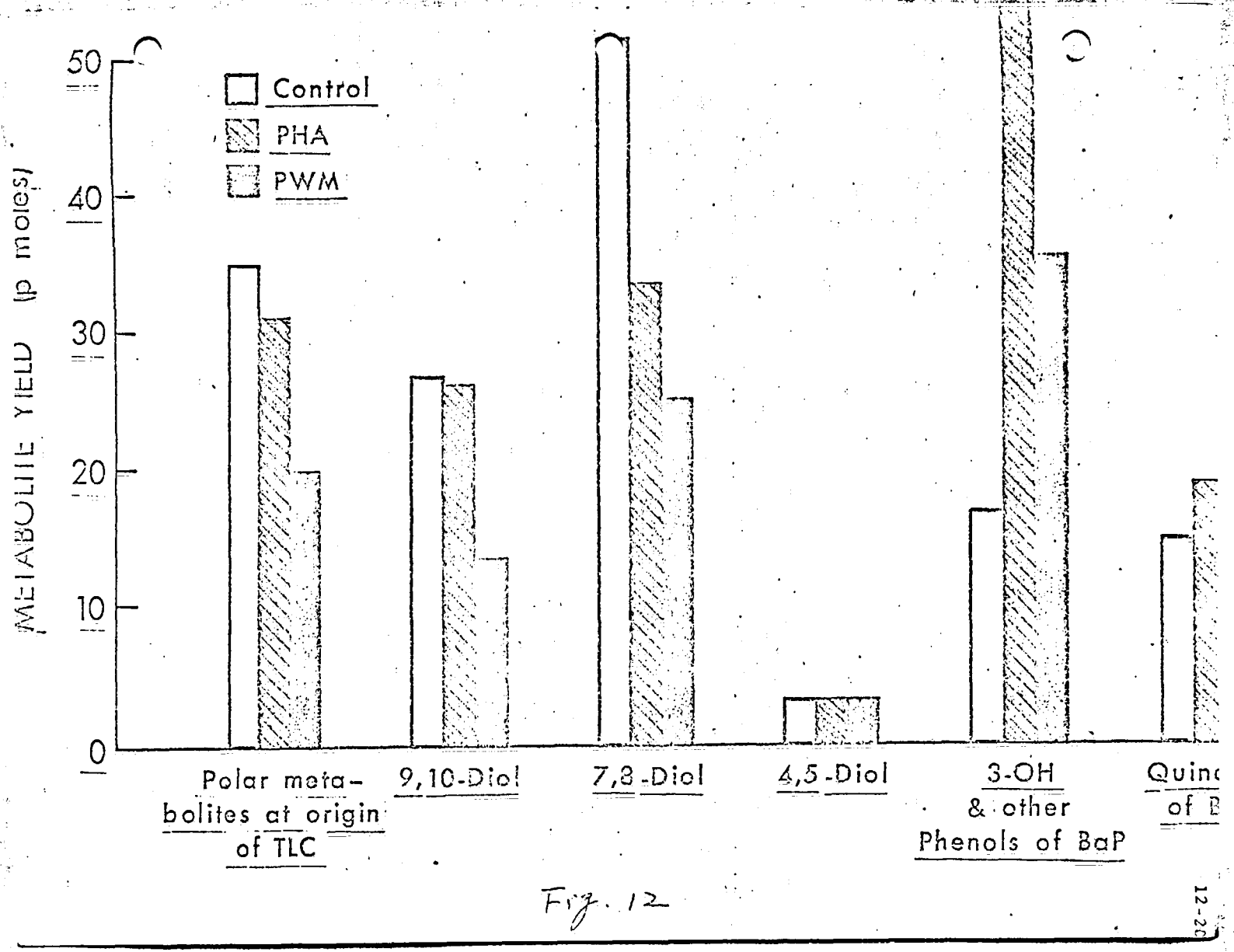

Fig. 12

12-20

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